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(54) Title: BACILLUS STRAIN AND ANTIBIOTIC SCREENING METHOD

(57) Abstract

A *Bacillus* strain has a chromosome with two reporter genes, a first reporter gene having a promoter which is dependent on active σ^F factor, and a second reporter gene having a promoter regulated similarly to the gene encoding the sigma factor. A method of using *Bacillus* strain in an assay for screening putative antibiotics.

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BACCILLUS STRAIN AND ANTIBIOTIC SCREENING METHOD

The assay method described herein targets a group of related activities functioning in cell division. The assay is based on the observation that activation of the sporulation-specific transcription factor σ^F, which has been extensively studied in several laboratories (reviewed by Errington, 1996, *Trends in Genetics* **12**, 31-34), requires the completion of cell division.

Synthesis of the sigma factor begins at the onset of sporulation but its product is initially held in an inactive state by the action of an anti-sigma factor, SpoIIAB. Release from inhibition requires the concerted action of at least two other proteins, SpoIIAA and SpoIIE, through a series of biochemical interactions that are now well characterised (Errington, 1996). These proteins serve to allow release of σ^F activity only after the sporulating cell has undergone asymmetric cell division and to restrict the σ^F activity to the smaller prespore cell type. This mechanism works in such a way that it renders σ^F activation dependent on septation. Thus, mutants or genetically engineered strains of *B. subtilis* that are prevented from undergoing septation because of the absence of essential cell division gene products such as *ftsZ* (Beall and Lutkenhaus, 1991, *Genes Devel.* **5**, 447-455), *divIC* (Levin and Losick, 1994, *J. Bacteriol.* **176**, 716-722) or *ftsL* (Daniel, R.A. and Errington, J., unpublished results), synthesise but do not activate σ^F. The dependence of σ^F activation on septation is herein used as the basis for a sensitive assay for inhibitors of cell division. Although the assay is based on inhibition of the specialised asymmetric cell division which occurs at the onset of sporulation, there is ample evidence that this process is functionally very similar to cell division in vegetative cells.

In one aspect the invention provides a *Bacillus* strain having two reporter genes, a first reporter gene having a promoter which is dependent on active sigma σ^F (or σ^E), and a second reporter gene whose promoter provides a measure of the synthesis of the (inactive) sigma factor.

In another aspect the invention provides a method of determining whether an agent inhibits cell division in *Bacillus* species, which method comprises inducing the *Bacillus* strain as described to sporulate in the presence of the agent, and observing expression of the first and second reporter genes. It is thought that the property of inhibiting cell division, is indicative of actual or potential anti-microbial properties in the agent. The method is thus expected to be useful for screening possible anti-microbial agents.

In another aspect the invention provides a method which comprises inducing the *Bacillus* strain as described to sporulate in the presence of an agent, observing expression of the first and second reporter genes and thereby determining that the agent inhibits cell division in the *Bacillus* species, and using the agent as an antibiotic to kill bacteria.

In yet another aspect the invention provides a method of killing bacteria which method comprises contacting the bacteria with a substance which does not inhibit σ^F (or σ^E) synthesis but does inhibit σ^F (or σ^E) activation.

The assay described below is based on use of σ^F activation but it could also have used σ^E, another sporulation specific sigma factor that is dependent on asymmetric septation (Stragier *et al*, 1988, Cell, **52**, 697-704). The dependence of σ^E on septation is now thought to be an indirect effect caused by the dependence of σ^E activity on σ^F activation (see Errington, 1996). Use of an σ^E-dependent reporter gene would be less desirable as it would probably detect more non-specific inhibitors than with σ^F.

Any *Bacillus* species may be used that is capable of sporulating under suitable conditions and for which genetic constructions can be made. *B. subtilis* is conveniently accessible and well characterised and is preferred.

The *Bacillus* strain constructed has a chromosome with two reporter genes each linked to a different promoter. A reporter gene is one which on expression gives rise to an easily detected or observed phenotype. For example, the expressed protein may be an enzyme which acts on a substance to give a product that is easily observed e.g. because it is coloured or chemiluminescent or fluorescent. Reporter genes capable of being expressed in *Bacillus* species are well known and documented in the literature. The two reporter genes are preferably chosen so that their products can be readily assayed simultaneously. *lacZ* has been used for more than ten years with great success in *B. subtilis*. There are a range of useful substrates that generate coloured or fluorescent products upon hydrolysis by β-galactosidase. The *uidA* gene of *E.coli*, also known as the *gusA* gene, has recently been harnessed for similar purposes, and the range of substrates available for the gene product, β-glucuronidase, is similar to that of β-galactosidase.

In a preferred form, the assay uses a specific strain of *B. subtilis* containing two reporter genes. The first (*gpr-uidA*) provides a means of monitoring σ^F (or σ^E) activity: its promoter is σ^F (or σ^E)-dependent and it directs the production of an enzyme, β-glucuronidase, the activity of which can be readily measured by spectrophotometry or spectrofluorimetry. The second reporter gene (*spoIIA-lacZ*), which monitors expression of the gene encoding σ^F (or σ^E), e.g. by virtue of having a promoter regulated similarly to the gene encoding sigma factor, provides a check for non-specific effects on sporulation or general inhibitors of gene expression. Again the product of the reporter gene is an

enzyme, β-galactosidase, that can readily be measured. By using appropriate (enzyme) substrates, the two enzyme activities could be measured simultaneously.

To use the assay, this *B. subtilis* strain would be induced to sporulate by the resuspension method (Sterlini and Mandelstam, 1969, *Biochem. J.* **113**, 29-37; Partridge and Errington, 1993, *Mol Microbiol* **8**, 945-955). The culture would be dispensed into the wells of a microtitre plate just before the onset of asymmetric cell division (e.g. after 1 h at 37°C). Individual wells would contain one or more potential inhibitors. After a period of incubation sufficient for induction of the *spoIIA* operon and activation of σ^F (or σ^E), the microtitre plate cultures would be assayed for the two reporter activities by standard methods. Potential "hits" would show inhibition of β-glucuronidase activity but normal β-galactosidase activity (indicating synthesis but not activation of σ^F (or σ^E)).

Alternatively, test compounds can be dropped onto a lawn of sporulating cells on a solid surface (e.g. agar). In this case, the effect of the test compounds on reporter gene activity will be assessed by the colour or fluorescence produced by hydrolysis of colourigenic or fluorogenic substrates incorporated into the solid medium.

Two kinds of compounds might be expected to be detected by the assay. First, the desired compounds that inhibit asymmetric cell division. Second, compounds that interfere in some way with the protein-protein interactions, or the kinase or phosphatase activities known to be involved in σ^F (or σ^E) regulation. These would be of purely academic interest (at least in the short term). The two classes could be readily distinguished by light microscopy because the latter class should form normal asymmetric septa.

Irrespective of the specific biomolecule affected in the screen, any compounds identified would be good potential candidates for development as antimicrobial agents because cell division is such a central

target. Moreover, since cell division proteins tend to be highly conserved in bacteria, it is likely that broad spectrum inhibitors could be obtained.

Reference is directed to the accompanying drawings in which:-

Figure 1 is a graph showing the effect of depletion of FtsZ on expression of *spoIIAA-lacZ* and *gpr-uidA*;

Figure 2 is a set of four graphs A, B, C, and D, showing detection of antibiotics affecting cell wall synthesis or cell division;

Figure 3 is a graph showing use of 96 well microtitire plates to induce sporulation and reporter gene expression in a format suitable for high throughput screening.

The following examples illustrate the invention.

Example 1

To illustrate the utility of the assay, a strain was constructed, containing the two reporter genes mentioned above (i.e., *gpr-uidA* and *spoIIAA-lacZ*) but in addition, a genetic insertion which renders the essential cell-division gene *ftsZ* dependent on an inducer chemical IPTG (Beall and Lutkenhaus, 1991). The strain was induced to sporulate under conditions in which the inducer was either present or absent and the two reporter activities were measured. Figure 1 shows the effect of depletion of FtsZ on expression of *spoIIAA-lacZ* (circles) and *gpr-uidA* (squares). Filled symbols indicate the reporter enzyme activities in the presence of FtsZ, and open symbols, in the culture from which it was depleted by removal of the inducer, IPTG. As shown, in the presence of inducer, both reporter genes were strongly induced 1 to 2 h after the onset of sporulation, whereas in the absence of the inducer, resulting in inhibition of cell division, only the *spoIIAA-lacZ* reporter, producing β-galactosidase, was activated. Because σ^F activity normally leads to repression of its own gene during sporulation, *spoIIAA-lacZ* expression is actually enhanced in

the absence of active σ^F. Samples taken about 180 min after the initiation of sporulation and assayed for β-galactosidase and β-glucuronidase would thus readily detect specific inhibition of σ^F activity. Note that in the absence of functional FtsZ, *gpr-uidA* activity is abolished (indicating that σ^F does not become active), whereas *spoIIAA-lacZ* expression (leading to σ^F synthesis) is enhanced, presumably because σ^F activity normally leads to repression of its own gene later in sporulation.

Example 2

To show that the assay method could detect actual inhibitors of cell division, the experiments shown in Fig. 2 were performed. The bacterial strain used (846) carried the following genetic markers: *trpC2 Ω(amyE::gpr-uidA aphA-3) (ϕ105J19) spoIIAA-lacZ cat.* Thus, β-galactosidase (from the *lacZ* gene) provides an indication of σ^F synthesis and β-glucuronidase (from the *uidA* gene) a measure of σ^F activation (dependent on cell division). The strain was induced to sporulate by standard methods (Partridge and Errington, 1993, Mol. Microbiol. **8**, 945-955). Immediately after induction, the culture was divided into several portions, which were treated with different known antibiotics. In the control (untreated) culture (panel A), β-galactosidase (filled squares) and β-glucuronidase (open squares) activities produced from the two reporter genes followed their normal kinetics (Errington and Mandelstam, 1986, J. Gen. Microbiol. **132**, 2967-2976; Partridge and Errington, 1993) (see also Fig. 1). In the presence of antibiotics that affect cell wall synthesis or cell division (bacitracin [50 μg/ml; panel B] and carbenicillin [110 μg/ml; panel C]) σ^F synthesis occurred, as indicated by accumulation of β-galactosidase with near normal kinetics, but its activation was blocked, as indicated by the elimination of β-glucuronidase activity. In contrast, with a general inhibitor of protein synthesis, erythromycin (50 μg/ml), both reporter genes were blocked (panel D).

Note that in the case of bacitracin (and vancomycin; data not shown), which affects cell wall synthesis generally, addition of the inhibitor at the concentration indicated caused a slight reduction in expression of the *spoIIAA-lacZ* reporter (as well as a block in *gpr-uidA* expression). It is likely that such a reduction in expression would not occur with inhibitors of proteins required specifically for formation of the septum, though no such inhibitors are presently available. Thus, of test chemicals that block *gpr-uidA* expression, those giving the highest levels of *spoIIAA-lacZ* expression would be most likely to affect targets required specifically for septation. Nevertheless, compounds giving a partial reduction in *spoIIAA-lacZ* expression might still be of commercial interest, as functions involved in cell wall synthesis are likely, in general, to provide the necessary selective toxicity needed for good antibiotics.

Note also that in the case of carbenicillin, cell lysis, as indicated by a fall in culture optical density (OD_{600}), began about 2 to 3 hours after the initiation of sporulation. Although this lysis probably occurs too late to explain the large difference in reporter activity, it provides an indication of a possible source of "false positive" results that might arise when the assay is put into practice. Since the *gpr-uidA* reporter is turned on about 1 hour later than the *spoIIAA-lacZ* reporter, it is possible that agents causing slow killing of the cells would have differential effects on expression of the earlier and later reporter genes. Agents having this effect through cell lysis could be detected and excluded, if necessary, by measuring the optical density of the culture about 3 or 4 hours after initiation of sporulation.

Example 3

To confirm that the results obtained above with batch grown *B. subtilis* strains could be obtained for cells grown in a microtitre plate format, cells of strain 846 (see above) were induced to sporulate by the normal method involving resuspension in a starvation medium (Partridge and Errington, 1993). Immediately after resuspension, the culture was divided into two portions. One portion was allowed to continue sporulating in the glass flask, as usual (filled symbols in Fig. 3). The other half of the culture was dispensed, in 50 µl aliquots, into the wells of a standard 8 by 12 microtitre plate with 360 µl wells (open symbols). The microtitre plate was incubated alongside the glass flask, shaking at 37°C. At intervals, the contents of one well of the plate and 50 µl of the flask culture were removed and assayed for β-galactosidase (circles) and β-glucuronidase (squares). As shown in Fig. 3, the time courses showed good induction of both reporter enzymes in the microtitre plate sporulated culture, though there were minor qualitative differences in their time courses from those of the flask culture. Cells induced to sporulate in microtitre plates were also observed to form spores with similar efficiency to those in flasks. We conclude that the sporulation experiments required for the assay to be run in high throughput mode can be done in readily available microtitre plates.

CLAIMS

1. A *Bacillus* strain having a chromosome with two reporter genes, a first reporter gene having a promoter which is dependent on active σ^F or σ^E factors, and a second reporter gene having a promoter regulated similarly to the gene encoding the sigma factor.

2. A *Bacillus* strain as claimed in claim 1, wherein the promoter of the first reporter gene is that of the *gpr* gene and the promoter of the second reporter gene is that of the *spoIIAA* gene.

3. A *Bacillus* strain as claimed in claim 1 or claim 2, wherein the reporter genes are *lacZ* and *uid*A.

4. A *Bacillus* strain as claimed in any one of claims 1 to 3, which is a *B. subtilis* strain.

5. A method of determining whether an agent inhibits cell division in *Bacillus* species, which method comprises inducing the *Bacillus* strain of any one of claims 1 to 4 to divide asymmetrically, as during sporulation, in the presence of the agent, and observing expression of the first and second reporter genes.

6. A method as claimed in claim 5, wherein the two reporter genes are expressed as enzymes, the activities of which are observed by fluorimetry or spectrophotometry.

7. A method as claimed in claim 5 or claim 6, wherein the *Bacillus* strain is induced to sporulate and is contacted, just prior to asymmetric cell division, with the agent.

8. A method as claimed in any one of claims 5 to 7, wherein, in a case where expression of the first reporter gene is observed to be reduced relative to the second reporter gene, the cells are examined to determine whether normal asymmetric septa have formed.

9. A method which comprises inducing the *Bacillus* strain of any one of claims 1 to 4, to sporulate in the presence of an agent, observing expression of the first and second reporter genes and thereby determining that the agent inhibits cell division in the *Bacillus* species, and using the agent as an antibiotic to kill bacteria.

10. A method of killing bacteria which method comprises contacting the bacteria with a substance which does not inhibit σ^F (or σ^E) synthesis but does inhibit σ^F (or σ^E) activation.

Fig.1.

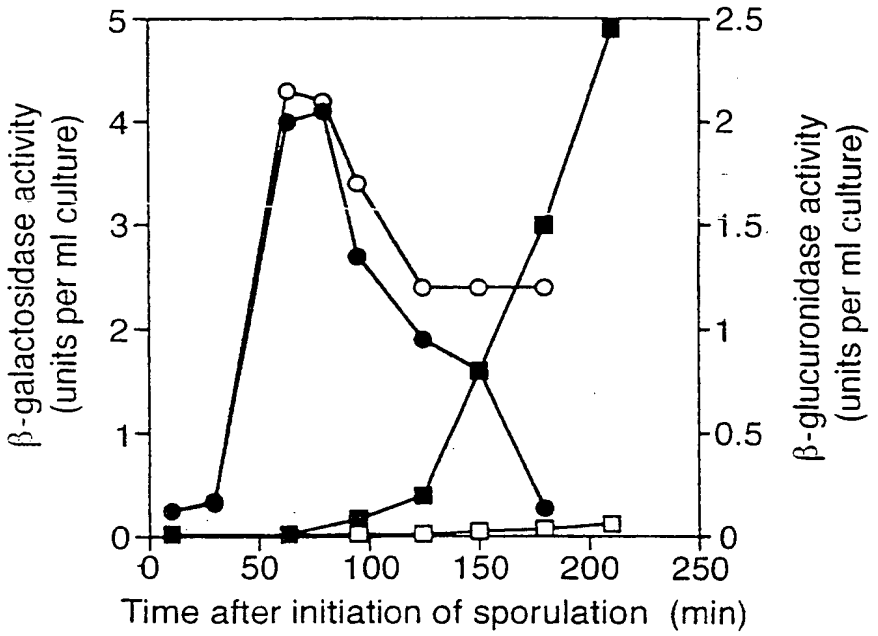

β-galactosidase activity (units per ml culture)
β-glucuronidase activity (units per ml culture)
Time after initiation of sporulation (min)
0
50
100
150
200
250
1
2
3
4
5
0.5
1.5
2.5

Fig.3.

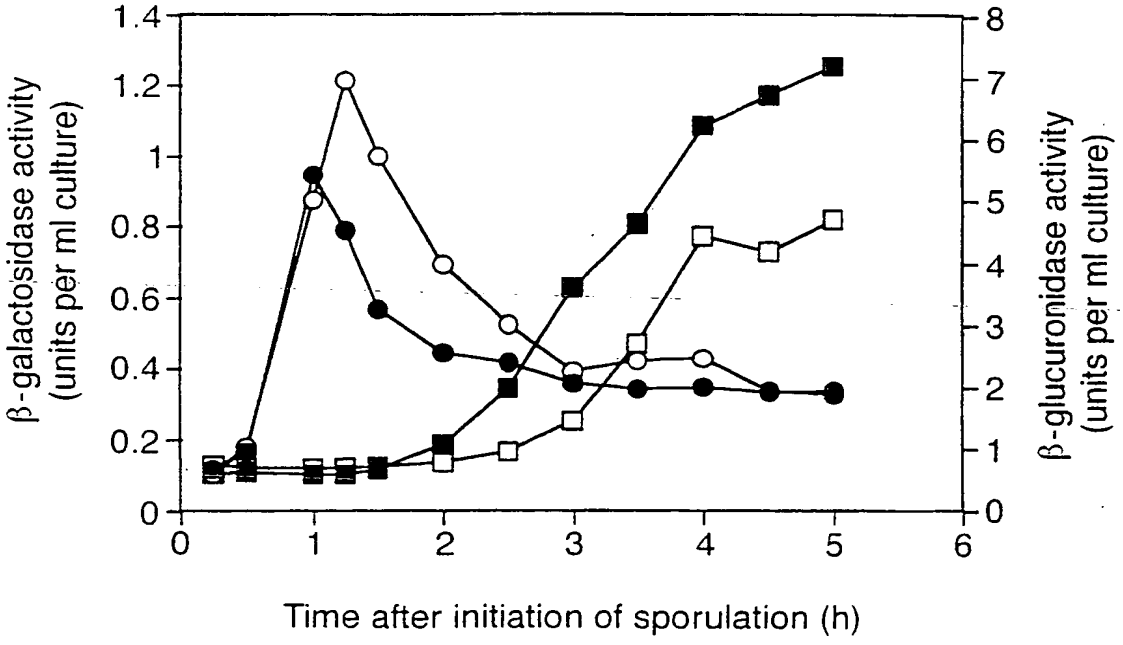

β-galactosidase activity (units per ml culture)
β-glucuronidase activity (units per ml culture)
Time after initiation of sporulation (h)
0
1
2
3
4
5
6
0.2
0.4
0.6
0.8
1.2
1.4
7
8

Fig.2A.

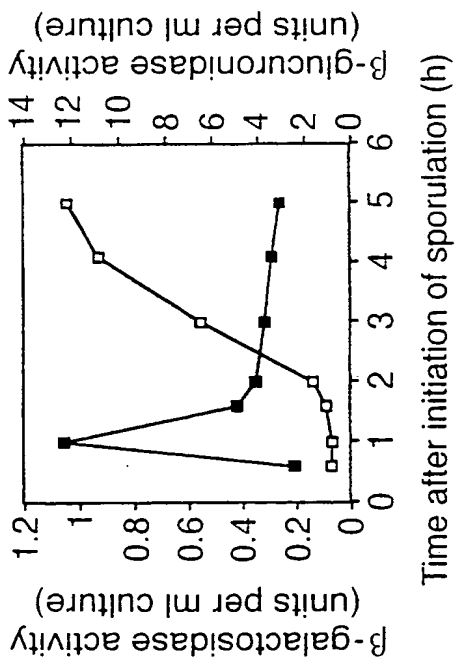


Fig.2B.

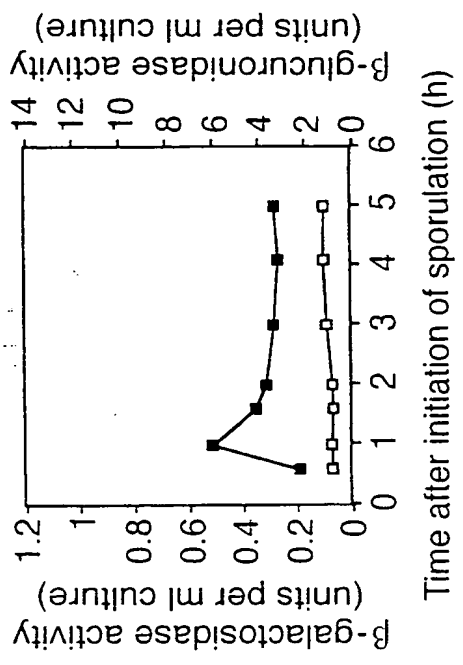


Fig.2C.

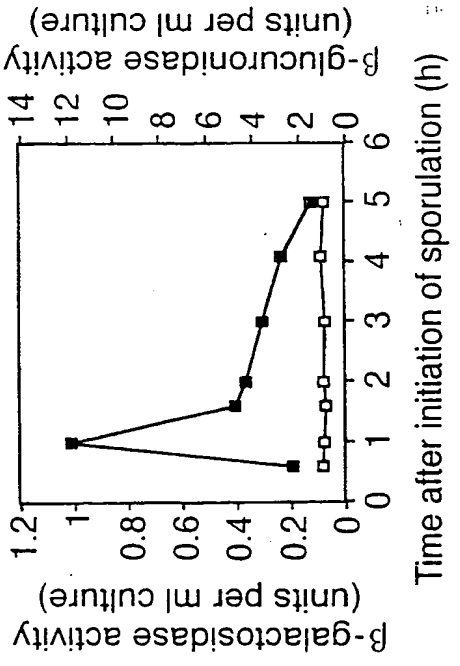
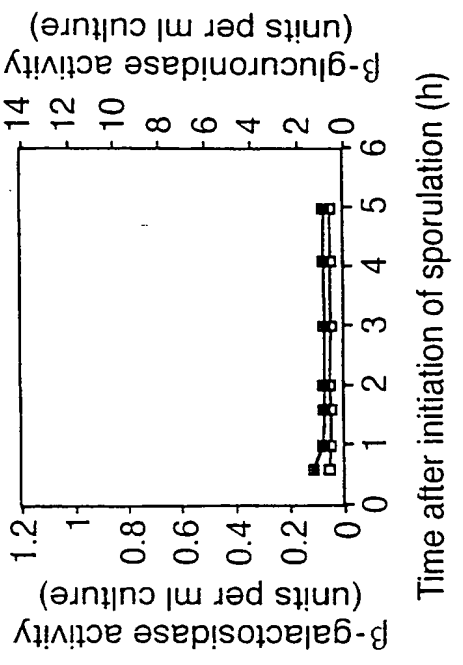


Fig.2D.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/03414

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/18 C12N15/65 //C12N1/21,C07K14/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	DONGXU SUN ET AL: "EFFECT OF CHROMOSOME LOCATION OF BACILLUS SUBTILIS FORESPORE GENES ON THEIR SPO GENE DEPENDENCE AND TRANSCRIPTION BY EOF: IDENTIFICATION OF FEATURES OF GOOD EOF-DEPENDENT PROMOTERS" JOURNAL OF BACTERIOLOGY, vol. 173, no. 24, December 1991, WASHINGTON US, pages 7867-7874, XP000601088 see page 7868 - page 7869; table 1 ---	1-7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 March 1998	16.04.98

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	STRAGIER P ET AL.: "Processing of a sporulation sigma factor in Bacillus subtilis: how morphological structure could control gene expression" CELL, vol. 52, 11 March 1988, NA US, pages 697-704, XP002060225 cited in the application see page 701, column 2 - page 702, column 2	1,5,9,10
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/03414

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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